SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELLING CHEMICAL AND BIOLOGICAL MACROMOLECULES

This invention refers to the chemical synthesis of a new paramagnetic β-amino acid derivative containing the stable nitroxide radical moiety inserted in its pyrrolidine structure and the 9-fluorenylmethyloxycarbonyl group (Fmoc) was chosen as its amine function protecting group. This paramagnetic compound is therefore a new type of spin probe (or spin label) and it can be used as alternative report molecule for labeling peptide sequences, other macromolecules and systems where the electron paramagnetic resonance spectroscopy (EPR) can be applied. The use of this compound can be extended also for other spectroscopic methods such as fluorescence and nuclear magnetic resonance since its paramagnetism may affect the spectra of this methodologies. Due to the presence of both carboxyl and amine groups in its structure, the use of this organic compound may be extended for labeling a great variety of other molecules or systems containing reactive function for these two groups.

The intermediate for the synthesis of the amino acid derivative of the present patent contains the structure 2,2,5,5-tetramethylpyrrolidine-1-oxil-3-amine-4-carboxylic acid henceforth denominated as Poac, synthesized more than two decades ago (v.g. Tetrahedron 491-499 [1965] and Bull. France, 3, 815-817 [1967]). Thus, the Poac derivative containing the Fmoc protecting group (2,2,5,5-tetramethylpyrrolidine-1-oxil-3-(9-fluorenylmethyloxicarbonyl)-amine-4-carboxylic acid) is the novel spin probe derivative of the present patent of invention. This new compound allows the POAC insertion as an usual amino acid at any position of the peptide sequence and its denomination will be

Fmoc-Poac or EPM-5 in this descriptive report. The chemical structure of this paramagnetic molecule is represented in **Figure 1**.

The electron paramagnetic resonance (EPR) [*in* Biological Magnetic Resonance, Berliner, L. J. and Reuben, J., eds, Plenum Publishing, New York, (1989)], is a modern and very useful spectroscopic method because it allows studies of any paramagnetically labeled macromolecules or biological systems regarding their conformation, mobilities, inter - or intra-molecular interactions, structuring state, etc. The wide spectrum of EPR application is already detailed in the literature (v.g. Free Nitroxyl Radical, Rozantsev, E.G., Ulrich, H., ed., Plenun Press, London, 1970), where a great variety of spin labels, i.e., chemical compounds which are paramagnetic due to the presence of an unpaired electron in its structure, is listed. They are therefore a class of free radical but must be necessarily stable towards normal temperature and physiological pH and also allow several chemical reactions or experiments without affecting its free radical moiety.

Amongst the most commonly used spin labels one can detach the nitroxide group-containing molecules and where the unpaired electron locates. The most significant progress in the RPE field for labeling of relevant biological structures such as peptides and proteins was achieved with this class of spin probes. Almost two decades ago appeared the first RPE application in the solid phase peptide synthesis methodology [(The Peptides: Analysis, Synthesis and Biology, vol. 2, Academic Press, New York, (1980)]. This approach was introduced by our group using instead, an other nitroxide-containing spin label denominated at that time as **Toac** (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine-4-carboxylic acid protected at it amine function with the acid labile tert-butiloxicarbonyl (Boc) group [(v.g. Braz. J. Med. Biol. Res. *14*, 173, (1981) and Biochim. Biophys. Acta, *742*, 63,

(1983)]. Thus the Boc-Toac spin probe was the first in the literature used to label a peptide sequence as an amino acid. However, due to chemical particularities of the peptide synthesis methodology, it was only possible to couple the Toac group at the peptide N-terminal position. To overcome this shortcoming, an alternative strategy was published by us which finally allowed the insertion of the spin label internally to the peptide sequence [v.g. J. Am Chem. Soc.*115,* 11042 (1993)].

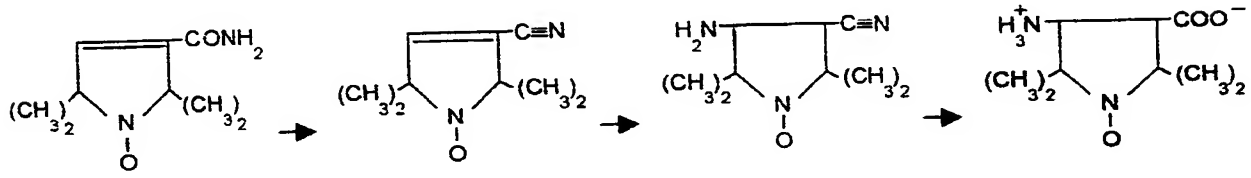
An impressive increase in the application of RPE for peptide chemistry field was further observed with reports investigating peptide conformational properties [v.g. J. Am. Chem. Soc. 117, 10555 (1995); FEBS Lett. 375, 239 (1995); Biopolymers 42, 821 (1997)] or of peptidil-resin solvation (Tetrahedron Lett. 375, 239 [1995]; Biopolymers 42, 821 [1997]). As an amino acid, TOAC was introduced in different positions of some biologically active peptides such as angiotensin II and bradykinin but a partial or integral loss of their biological potencies were observed due to the introduction of a non natural compound in their structures. [Peptides 1996, R. Ramage and R. Epton, eds, Mayflower Scientific Co. p. 673, (1998)].

However, we recently described the synthesis of a peptide hormone labeled with Toac and where its biological potency was entirely preserved. [v.g FEBS Lett. *446,* 45 (1999)]. This result was obtained with the tridecapeptide α-melanocyte stimulating hormone analogue and owing to its potentialities in a great number of chemical-biological assays (this analogue is paramagnetic, naturally fluorescent and fully active), was submitted for the patent process (PI 9900595, February 24, 1999 : "Synthesis of the first paramagnetic and active α-melanocyte stimulating hormone analogue containing free radical amino acid".

In spite of these results, one still remaining shortcoming to the use of Toac in peptide chemistry refers to the severe difficulty in coupling the subsequent amino acid residue of the peptide sequence during the synthesis. It seems to

be due to the low nucleophilicity of Toac amine group whose pKa of 8 (when in free state) decreases to about 5.5 when bound to the N-terminal portion of a peptide chain [v.g Braz J. Med. Biol. Res. *14*, 173 (1981) and Biochim. Biophys. Acta. *742*, 63 (1983)]. Several recouplings and the increase on the temperature of the coupling reaction are usually necessary to assure complete incorporation of the subsequent amino acid of the desired peptide sequence [J. Am. Chem. Soc.*115*, 11042 (1993)].

Aiming to overcome this limitation inherent to the Toac probe and searching for an alternative spin label which may induce a differentiated conformational constraints in peptide structures, we decided to synthesize the **FMOC-POAC** according to partially described synthetic route shown below [Tetrahedron, 491-499 (1965); Bull. Soc..Chim. France, *3*, 815 (1967)] :



P1 **P2** **P3** **P4**

Step # 1 - Synthesis of 2,2,5,5-tetrametilpyrroline-1-oxil-3-cyano (P2)

This product was synthesized by treating the compound P1 (from Sigma Co) with tosil-chloride in dry pyridine. To 28.7 g (1.5×10^{-1} mol) of tosil-chloride, 15.3 g (8.35×10^{-2} mol) of P1 dissolved in 100 mL of dry pyridine was added and left at room temperature for 48 hours. After this period, 10 g of KOH dissolved in 250 mL of water were added and the mixture was heated up for 80°C. After cooling, the product was extracted with sulfuric ether, washed with diluted HCl, diluted $NaHCO_3$ solution, water and dried over anhydrous Na_2SO_4. After evaporation of the solvent under reduced pressure 12.76 g (yield = 92%) of an orange powder was obtained and further purified in an

alumina column using benzene as eluent. The product (P2) showed a single spot in thin layer chromatography and with following characteristics: M.P. = 62-63°C, M+ = 165; Elementary analysis: found: C, 65.36% , H, 7.50% e N, 17.10 % ; calculated : C, 65.43% , H, 7.93% e N, 16.96%).

Step # 2 : Synthesis of 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amine-4-cyano (P3).

In a 3 L round bottom flask, 600 mL of liquid ammonia, 9 g (5.44×10^{-2} mol) of P2 and 120 mL of water were added. The flask was tightly closed to maintain the mixture under pressure and left at room temperature. After 3 days, the ammonia was eliminated and the product extracted with chloroform. The crude product crystallized from ether-petroleum ether yielded 9.37 g (yield = 94%) of a yellow powder with the following characteristics: M+ = 182; P.F. 84-85 °C; Elementary analysis: found: C 58.98%; H, 8.37%; N, 22.21%; calculated ($C_9H_{16}ON_3$): C, 59. 31%; H, 8.85 % ; N, 23.06 %).

Step 3 - Synthesis of 2,2,5,5-tetrametilpyrrolidine-1-oxil-3-amino-4-carboxylic-acid (P4) -POAC.

8 g (4.38×10^{-2} mol) of P3 and 40 g of $Ba(OH)_2$ were suspended in 600 mL of water and added to a 3 L round bottom flask. The flask was tightly closed and heated up to 120°C for 90 minutes. After cooling, the mixture was neutralized with excess of dry ice and filtered. The aqueous solution was concentrated under reduced pressure and yielded 8 g of crude product (yield: 90%) that was crystallized from 90% ethanol. The product presented the following characteristics: M.P. = 212°C (melts with sublimation); M+ = 201 (**Figure 2**), single pick in HPLC, (**Figure 3**); elementary analysis : found : C, 53.1% ; H, 8.28 %; N, 13.95 % ; calculated ($C_9H_{17}N_2O_3$): C, 53.71 % ; H, 8.52; N, 13.92 Infra-red (KBr) : cm^{-1} : 3084, 2872, 2792, 2548 and 2132 (NH+3); 1643 ν_{AS} (NH+3) ; 1574 (ν_{AS} , C=O); 1456 (δ CH3); 1396 and

1376 (gem-dimethyl and COO-); 782 (δ C=O). The **Figure 4A** shows the EPR spectrum of POAC in aqueous solution, pH 5. The calculated values for the two rotational correlation times (τ_B and τ_c) were 0.509 x 10^{-10} and 0.597 x 10^{-10},respectively.

Preparation of Fmoc-Poac

201 mg (1 mmol) of P4 was dissolved in 1.5 mL of water in presence of 286 mg of sodium carbonate.10 H_2O in which 337 mg (1mmol) of Fmoc-succinimidyl-carbonate dissolved in 1.5 mL of acetone was added drop-wise. The reaction was carried out at room temperature with stirring and the pH was maintained around 9 by addition of sodium carbonate. After 3 hours, the mixture was diluted with 25 mL of water, acidified with 1 N HCl until pH 2. The desired product was extracted with ethyl acetate, washed with small portions of water, dried over anhydrous sodium sulfate. After filtration, the solvent was evaporated under reduced pressure. The crude product was crystallized twice with chloroform and yielded 380 mg (yield : 90%). The product was characterized by mass and infra-red spectroscopy, elementary analysis and EPR. Characteristics: M+ = 423 **(Figure 5)**; elementary analysis : found: C, 67.9 % ; H, 6.35 %; N, 6.60 ; calculated ($C_{24}H_{27}O_5N_2$): C, 68.08% ; H, 6.28 % ; N, 6.62%; IR (KBr) cm^{-1}: 3444-3338 (broad band OH and -CONHR); ~3030 (νAr CH); 3000-2700 (νAr COOH); 1723 (R-O-C-ON- and COOH); 1543 (δ_{NH} and νCN); 1450 (δ CH_3); 1235-1150 (gem-dimethyl group). The EPR spectra of Fmoc-POAC in dimethylformamide is represented in **Figure 4B** and the calculated τ_B and τ_c values are 1.14 x 10^{-10} s.rad^{-1} and 1.79 x 10^{-10} s. rad-1, respectively.

Synthesis of Poac^7-angiotensin II

Angiotensin II analogue labeled with the spin probe POAC (Asp-Arg-Val-Tyr-Ile-His-Poac-Phe) was synthesized in 0.15 mmol scale, by the solid phase

method already mentioned and with alteration to provide the insertion of this marker in the middle of the peptide chain. [J. Am. Chem. Soc. *115*, 11042 (1993)]. FMOC-Phe-Wang-resin [J. Am. Chem. Soc. *95*, 1328 (1973)] with 0.41 mmol/g substitution degree acquired commercially was used. All couplings were carried out using 2.5 fold excess for the FMOC-amino acids and 3 fold excess for Fmoc-POAC. The acylating reagents for coupling were diisopropylcarbodiimide (DIC) and 1-hidroxibenzotriazole in dichloromethane : dimethylformamide mixture (1:1,v/v) as solvent. The Fmoc deprotection was performed with 20% piperidine in dimethylformamide (v/v) for 20min.

Interestingly, this synthesis demonstrated that the pyrrolidine structure of the Poac spin label allowed much easier incorporation of the subsequent amino acid residue than the observed during the Toac^7-All synthesis. In this latter case, repetitive recoupling reactions were necessary including the increase in the temperature. These procedures were not necessary in the case of Poac derivative thus demonstrating that the Poac amine group reactivity is much higher than that of Toac.

After the completion of the synthesis, the peptide was cleaved from the resin in anhydrous HF containing 10% of the p-cresol and dimethylsulfide mixture, at 0°C for 90 min. The crude peptide obtained after extraction and lyophilization (125 mg) was dissolved in 70 mL of water and the pH was raised to 10 with ammonium hydroxide and stirred for 2 h, to revert the nitroxide protonation that occurs during the HF treatment. After lyophilization, the peptide was purified by preparative HPLC (high performance liquid chromatography) using a reverse phase C_{18} (25 x 250 mm) and ammonium acetate 0.02 M, pH 5 and acetonitrile 60% in water, as solvents A and B, respectively. The linear gradient applied was from 20-65% of B in 135 min.

The homogeneity of the peptide was confirmed through analytical HPLC (**Figure 6**), mass spectrometry, M+ = 1132.55 (**Figure 7**) and the amino acids analysis showed the expected composition : Asp 0.95 (1.00); Val 0.96 (1.00); Ile 1.20 (1.00); Tyr 1.02 (1.00); Phe 1.00 (1.00); His 0.96 (1.00); Arg 1.02 (1.00). The **Figure 8** displays the EPR spectra of 0.25 mM Poac^{7}-All at pH 3, 6 and 9 aqueous solution. No significant variation on the rotational correlation time values was observed for this paramagnetic All analogue thus suggesting that its conformations is not affected by the pH of the media.